

THE USE OF PURE LIPOIDS AND ALCOHOLIC EXTRACTS WITH ACTIVE AND INACTIVE SERUM IN THE COMPLEMENT-FIXATION TESTS FOR SYPHILIS*

THOMAS MAC RAE, M.D., A. B. EISENBREY, M.D., AND
HOMER F. SWIFT, M.D.

NEW YORK

In a comparative study of the Wassermann and Noguchi reactions reported by one of us¹ last year, it was stated that by the Noguchi method many positive reactions were obtained with non-specific serums. The same serums, when they were not inactivated, also gave positive reactions by the Wassermann method. When, however, inactivated serum was used in both methods, these non-specific positive reactions disappeared. As a result of this work, in which an alcoholic extract of syphilitic liver was used as antigen in the same proportion in both methods, it was recommended that inactive rather than active serum be used in the Noguchi method.

Noguchi² has since shown that a non-specific fixation of complement occurs with unheated serum, when protein bodies are present in the antigen extract, and that this non-specific fixation does not occur with inactivated serum. He attributes the non-specific positive reactions, reported by Swift, to the fact that, in the alcoholic extracts used as antigen, protein bodies are present in varying proportions. To eliminate such non-specific reactions, he advises the use as antigen of an ether-soluble, acetone-insoluble organ extract which is free of protein.

It is the object of this communication to present the results of a comparative study of the use of the two forms of extract in both the Wassermann and Noguchi methods, the latter being performed with both active and inactive serum.

DESCRIPTION OF METHODS

Preparation of Antigens.—The Ether Extract: The pure lipid solution was prepared, after the method recommended by Noguchi, from

* From the Department of Pathology, the University and Bellevue Hospital Medical College, New York.

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1. Swift, H. F.: The Use of Active and Inactive Serum in Complement Deviation Test for Syphilis, *THE ARCHIVES INT. MED.*, iv, 494.

2. Noguchi, H.: *Proc. Soc. Exper. Med. and Biol.*, 1909, vii, 55.

the liver of a patient who presented evidence of visceral syphilis. The lipoid was twice fractionated with acetone. After the first fractionation the solution was found to be hemolytic, but after the second fractionation this hemolytic property disappeared. A saturated solution in ethyl alcohol of this pure lipoid was then made; the saturation was carried on for two days at 37 C., after which the preparation was filtered and kept as a stock solution.

The Alcoholic Extract: The liver from a case of congenital syphilis was extracted in absolute alcohol at 37 C. for one week. The filtrate of this extract was kept as a stock solution. At the end of six weeks it was noted that this alcoholic extract was becoming weaker in its power of fixation, so another, similarly prepared, was substituted. This retained its strength, and was used throughout the latter part of the series of tests here reported. From these alcoholic stock solutions fresh emulsions in normal saline solution were made each day.

Standard of Fixation Power: In preparing and comparing different extracts, used as antigen, it is obviously desirable to have their power of complement-fixation as nearly equal as possible. This is necessary not only for purposes of comparison in any given set of tests, but also, when the effect of treatment is studied over a period of years, in order that new antigens of similar power may be prepared from time to time. The following method has, therefore, been devised for the preservation of a standard of fixation power:

With an efficient antigen of known standard the smallest amount of syphilitic serum that will completely fix the unit of complement is determined. This is called the syphilitic unit. The quantity of syphilitic serum constituting this unit and the unit of the same complement are mixed with decreasing amounts of the new antigen, and thus the minimum amount of the antigen that will completely fix one unit of complement in the presence of one unit of syphilitic antibody is determined. Such determinations are made with a number of syphilitic serums before the final standard is fixed, and this is further controlled by using several negatively reacting serums. In this way the emulsions of the antigens used in the reactions may be brought to equal fixation power.

Wassermann's Method.—Quantities one-half of those usually described were used: inactivated human serum, 0.1 c.c.; guinea-pig serum, 0.05 c.c., made up to 0.5 c.c. with normal saline solution; antigen, standard amount made up to 0.5 c.c.; total volume, 1.5 c.c. After incubation for one hour, 0.5 c.c. of a 5 per cent. suspension of sheep cells and two units of antish sheep hemolysin made up to 0.5 c.c. are added, and incubation

continued for one hour. The reactions are controlled by the use of a known positive and a known negative serum. The reagents are controlled by combining them in the usual proportions, omitting human serum in one tube and antigen in another. In addition it was determined whether 0.05 c.c. of each suspected serum would hemolyze 0.5 c.c. of the sheep cell suspension in the presence of 0.05 c.c. guinea-pig serum; if, in this control, hemolysis is complete at the end of one hour, it is evident that 0.1 c.c. of the serum contains at least two units of antish sheep hemolysin. When this amount of native hemolysin was present, no rabbit's hemolytic serum was added, and it was possible to avoid an excess of hemolysin with a consequent masking of a partial fixation. In all reactions the hemolytic unit was determined each day for both the antish sheep and anti-human hemolysins.

PROTOCOL

PART I. DETERMINATION OF SYPHILITIC UNIT *

Amount Known Positive Serum, c.c.	Complement, 10 Per Cent., c.c.	Standard Anti-gen Emulsion, c.c.	Procedure.	Result.
0.1	0.5	0.5	Total volume of all made 1.5 c.c.; incubate 1 hour;	++
0.05	0.5	0.5	add 0.5 c.c. 5% sheep cells	++
0.025	0.5	0.5	and 2 units antish sheep hemolysin; incubate 1 hour.	++
0.012	0.5	0.5		+
0.006	0.5	0.5		+-

PART II. DETERMINATION OF NEW ANTIGEN UNIT †

Amount Known Positive Serum, c.c.	Complement, 10 Per Cent., c.c.	New Anti-gen Emulsion, c.c.	Procedure.	Result.
0.025	0.5	0.6	Total volume of all 1.5 c.c.; incubate 1 hour; add	++
0.025	0.5	0.5	0.5 c.c. 5% sheep cells and	++
0.025	0.5	0.4	2 units antish sheep hemolysin; incubate 1 hour.	+
0.025	0.5	0.3		+-
0.025	0.5	0.2		-

* Usual controls of all reagents in each part of experiment.

† 0.5 c.c. of the new antigen equals 0.5 c.c. of the old antigen.

Noguchi Method, with Active Serum.—One capillary drop of suspected serum and 0.04 c.c. guinea-pig serum made up with normal saline solution to 0.4 c.c. were mixed with antigen in the same proportion as in the Wassermann reaction (0.4 c.c. of the freshly prepared emulsion). The total volume was made up to 1.2 c.c. and incubated at 37 C. for one hour, and then two units of anti-human hemolysin made up to 0.1 c.c., and 0.1 c.c. of a 10 per cent. suspension of human cells were added. Incubation was continued for another hour.

Noguchi Method with Inactive Serum.—After heating to 56 C. for thirty minutes the serum was used in amounts of 0.08 c.c. The other

reagents were used as in the test with active serum. In the control tube without antigen 0.1 c.c. of the serum was used. The reagents were controlled as described in the Wassermann reaction. The various reactions were all performed with the same complement, so that the results of each set of reactions are comparable.

The antigens were at first used in the same proportion in the Noguchi "active" as in the Wassermann and Noguchi "inactive" reactions. It was found, however, that for the Noguchi "active" reaction it was, as a rule, too strong. Although it was tried out against the serum of several normal persons and gave no reaction, it was found advisable to reduce the amount to three-fourths of that used in the "inactive" method. This gave sensitive reactions with syphilitic serums and did not give so many reactions in non-specific cases.

DISCUSSION OF METHODS

In the preparation of antigens the following points are important:

It is desirable: (1) that the extract react with the highest percentage of syphilitic serums and not react with non-syphilitic serums; (2) that it retain its properties unimpaired; and (3) that, with a constant method of preparation, an extract having the same properties be always obtained.

Alcoholic extracts of organs have been used most extensively thus far, and it is the general opinion that an antigen in this form retains a practically uniform strength for many months. Many who have tried the antigen-impregnated papers have noted that the character of their reaction changes after a time. Often the fixing power is much decreased and occasionally anticomplementary action is noted. In studies which are to extend over several months, and in clinics and practice where the effect of treatment on the reaction is to be noted, a single preparation of uniform strength is very desirable. Alcoholic extracts of organs vary in character in the widest degree. From some livers, extracts are obtained that are so hemolytic that they cannot be used. Other extracts may be very anticomplementary in proportion to their fixing power. The ether-soluble, acetone-insoluble solution offers the most efficient antigen. The ether extraction appears to eliminate the anticomplementary properties, and the acetone fractionation surely removes the hemolytic substances of the original alcoholic extract, leaving the lipoids, which are the efficient bodies in the complement-fixation test. It is probable, also, that by this method similar bodies are obtained from different livers, so that the variations noted in the solutions obtained by simple alcoholic extraction are avoided. For these reasons the Noguchi method of antigen preparation appears to be the most desirable one. Only an actual trial of the

two forms of antigen, such as is here presented, can give an idea as to their relative value.

DISCUSSION OF RESULTS

Tables 1, 2 and 3 represent the results of the comparative study of the Wassermann and the Noguchi reactions, the latter performed with both active and inactive serum, using both the alcoholic extract and an ether-soluble, acetone-insoluble extract as antigen in 300 consecutive specimens of blood obtained from hospitals, dispensaries, and private practice. The cases are classified according to the stages of syphilis indicated by the diagnoses accompanying the specimens. Cases of leprosy and of doubtful lesions, possibly syphilitic, but giving a positive reaction, are also shown in Table 2. In order that possible sources of error may be indicated, non-specific cases, in which there is little probability of syphilis, but in which weak positive reactions were obtained, are also given individually in Table 3.

TABLE 1.—SYPHILIS, ALL STAGES, TREATED AND UNTREATED; 212 CASES

Stages.	Cases	Antigen.	Wassermann				Noguchi Active				Noguchi Inactive			
			++	+	+-	-	++	+	+-	-	++	+	+-	-
Primary.....	12	I	5	1	4	2	8	3	1	0	5	2	2	3
		II	7	0	4	1	10	1	1	0	6	1	3	2
Secondary.....	44	I	29	5	6	4	38	4	0	2	27	3	8	6
		II	31	8	2	3	37	3	1	3	30	5	5	4
Tertiary.....	56	I	34	4	9	9	40	11	3	2	26	13	5	12
		II	37	4	8	7	41	9	4	2	31	8	6	11
Early Latent.....	31	I	8	2	9	12	12	9	6	4	3	3	10	15
		II	10	1	12	8	14	10	4	3	6	3	11	11
Late Latent.....	61	I	10	5	16	30	21	11	12	17	9	4	6	42
		II	12	5	15	29	22	8	11	20	10	6	6	39
Tabes.....	6	I	..	1	..	5	1	1	2	2	..	1	3	2
		II	..	1	..	5	1	1	2	2	..	1	3	2
Congenital.....	2	I	1	1	1	1	1	1
		II	1	1	1	1	1	1
Total cases.....	212													

In this study no attempt has been made to classify the cases according to the amount of antisyphilitic treatment received, and consequently numerous negative or weakly positive reactions are recorded among the cases classified as frankly syphilitic.

The superiority of the pure lipid over the alcoholic extract is noted in all the methods and in all the stages of syphilis. The Noguchi method with active serum gives the largest number of positive reactions. It appears earlier in the primary stage, and persists longer in the cases under treatment. The Wassermann method gave the highest number of positive reactions, and the Noguchi method with inactive serum the fewest. The difference in the two latter methods is largely one of degree;

TABLE 2.—SUSPECTED SYPHILIS AND LEPROSY; 21 CASES *

Diagnosis.	Wasser-mann.		Noguchi Active.		Noguchi Inactive.		Remarks.
	I A.E.	II P.L.	I A.E.	II P.L.	I A.E.	II P.L.	
Leprosy.....	--	--	+	+-	--	--	Anesthetic type. Nastin B injections.
Leprosy.....	--	--	+	+	--	--	Mixed type. Nastin B injections.
Leprosy.....	--	++	++	++	++	++	Nodular type.
Ulcerations at angle of mouth.....	--	--	--	++	--	--	Has had gonorrhea several times. Had 2 months' mixed treatment 4 years ago.
Cystitis, chronic.....	--	--	+	+	--	--	Had gonorrhea 3 times in 2 years. No mercury.
Chaneroid.....	--	+-	++	++	+-	+-	No symptoms except local. No treatment except local.
Intractable nose-bleed	--	--	+	+-	--	--	Following incision of membrane. Syphilis in family.
Paralysis of leg.....	--	--	+	+	--	--	
Stricture of rectum.....	--	--	++	++	--	--	New growth or specific stricture.
Epididymitis, chronic.	--	--	+	++	+	+	Chaneroid persisted 40 days after exposure. No secondaries.
Rash.....	--	--	+-	+-	--	--	Herpetetic eruption on penis 3 months ago.
Arteriosclerosis.....	--	--	+	+	--	--	Aortic insufficiency. Nephritis.
Psoriasis.....	--	--	+-	+-	--	--	Chaneroid 5 years ago. No secondaries. Took pills 2 months.
Headache.....	--	--	+	--	--	--	Worse at night.
Pellagra?; adenopathy	--	--	+-	--	--	--	Chancre? 4 years ago. Had 15 days' mixed treatment recently.
Epididymo-orchitis....	--	--	+-	+-	--	--	Two weeks.
Sore on mammary gland.....	+-	+-	+-	+-	--	--	Ten days. Very suspicious of chancre. No mercury.
Headaches and dizziness.....	--	--	+-	--	--	--	No mercury.
Cirrhosis of liver, ascites.....	--	--	++	++	--	--	Had gonorrhea.
Asthma and paralysis agitans.....	--	--	+	+	+-	--	Had gonorrhea twice.
Aortic regurgitation..	++	++	++	++	++	++	Had gonorrhea.

* No definite syphilitic history obtainable.

TABLE 3.—NON-SYPHILITIC, BUT GIVING SLIGHT REACTION; 17 CASES *

Diagnosis.	Wasser-mann.		Noguchi Active.		Noguchi Inactive.		Remarks.
	I A.E.	II P.L.	I A.E.	II P.L.	I A.E.	II P.L.	
Enlarged cervical glands.....	--	--	+	--	--	--	One month.
Psoriasis.....	--	--	+	+-	--	--	Yearly exacerbations 10 years.
Chaneroid, gonorrhea.....	--	--	+	--	--	--	Has had no mercury.
Scabies.....	--	--	+-	--	--	--	
General furunculosis.....	--	--	+-	+-	--	--	
Chronic abscess, abdomen.....	--	--	++	+-	--	--	Brother has syphilis.
Rheumatism-endocarditis.....	--	--	+-	--	--	--	
Varicocele.....	--	--	+	--	--	--	No mercury.
Acute bronchitis.....	--	--	+	+-	--	--	No mercury.
Actinomycosis.....	--	--	+-	+-	--	--	No mercury.
Adenopathy.....	--	--	+-	+-	--	--	General enlargement.
Neuritis.....	--	--	+	+-	--	--	Lead? 1 month. No. Hg.
Tumor of pelvis.....	--	--	+	--	--	--	Probably malignant.
Sclerodactylitis.....	--	--	+-	--	--	--	Fingers frozen 20 years ago.
Edema and congestion, left leg	--	--	+	+-	--	--	No mercury.
Condyloma acuminata.....	--	--	+	--	--	--	Of anus, 1 year.
Hodgkin's disease.....	--	--	+	--	--	--	Enlarged cervical glands 7 years.

* Fifty non-specific cases taken in hospital wards and dispensaries gave uniformly negative results.

TABLE 4.—ANALYSIS OF 300 CASES *

Character of Cases.	Number of Cases.	Antigen.	Wassermann.						Noguchi Active.						Noguchi Inactive.									
			++		+		+-		-		++		+		+-		-							
			No.		%		No.		%		No.		%		No.		%							
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%						
Syphilis, all stages, treated and untreated	212	Alc. ext.....	87	41	18	8	44	21	63	30	121	57	39	18	24	12	28	13	34	16	81	38		
		Pure lipoid.	98	46	19	9	41	12	54	26	126	59	32	15	23	11	31	15	34	39	24	16	70	33
Suspected syphilis with no syphilitic history. Leprosy, 3 cases.	21	Alc. ext.....	1	3	0	0	1	3	19	94	5	24	9	43	6	28	1	5	2	9	16	77		
		Pure lipoid.	2	9	0	0	2	9	17	82	7	35	5	24	6	28	3	13	2	10	1	5	17	80
Non-specific; 50 cases gave negative reactions throughout.	67	Alc. ext.....	67	100	1	1	8	12	8	12	50	75	67	100
		Pure lipoid.	67	100	8	12	59	88	67	100

* Percentages are computed on the number of cases in each group. The three types of reaction using two antigens were done on each case.

when weak reactions are present, the Wassermann method is easier to read, because there are more cells to indicate the amount of hemolysis. Attention has been called by one of us³ to the relative sensitiveness of the Wassermann and Noguchi methods in repeated examinations for determining the effect of treatment. This point is well illustrated in the table under latent syphilis. As a rule, the cases of latent syphilis have received more treatment than the cases of active syphilis. The sensitiveness of the active serum method is more marked in this stage than any other.

In the table of suspected cases (Table 2) it will be noted that, with the exception of leprosy, those individuals who were the most strongly suspected of syphilis gave the most marked reaction. In either method in which inactive serum was used, a positive reaction practically always was an indication of the presence of syphilis. With the active serum the cases in which there was the least ground for a suspicion of syphilis, the strongest reaction was obtained with the alcoholic extract. The oversensitiveness of the alcoholic extract is also well marked in the cases in which there was little evidence of the presence of syphilis. Weakly positive reactions in such cases, however, are often found with the pure lipid, so that weakly positive reactions have practically no diagnostic or prognostic value. The importance of the use of absolutely fresh serum must be emphasized, for positive reactions obtained with unheated serum three days old have practically no value. We have found it extremely important, as pointed out by Noguchi, to examine the specimens of suspected serum within twenty-four hours, as it has been found that specimens examined one day with negative results give a partial fixation on the following day. This fact, no doubt, explains the reason for a certain number of so-called non-specific reactions with the active serum. With the elimination of these apparent non-specific reactions by inactivating the serum, we feel that more reliable results will be obtained by the use of both active and inactive serum. A negative reaction with active serum gives strong evidence against active syphilis; while a positive reaction with inactive serum speaks more strongly for syphilis than one with active serum. It is, however, only fair to say that, with the pure lipid as an antigen, we have not seen a strongly positive reaction in any case, except leprosy, in which syphilis could be absolutely excluded. Reactions of less degree than "absolutely positive" always are to be regarded with some doubt, unless other symptoms of syphilis or history of the disease can be obtained.

3. Swift, H. F.: The Effect of Treatment on the Wassermann Reaction, *THE ARCHIVES INT. MED.* (to be published).

CONCLUSIONS

1. The "pure lipoid" antigen (ether extract) has proved the more satisfactory. It has given, with syphilis, the largest percentage of strongly positive reactions by all three methods.

2. The alcoholic extract antigen, when used with active serum, has given the largest percentage of non-specific reactions.

3. Inactivation apparently destroys the power of a non-specific serum to cause a positive reaction.

4. The Noguchi method, using active serum, gives the most sensitive reaction in syphilis; the Wassermann and the Noguchi "inactive" methods stand in the order named.

5. One of the most important factors in securing reliable results, when active serum is employed, is the performance of the reaction within twenty-four hours after the serum is obtained.

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271 West Seventy-Third Street—338 East Twenty-Sixth Street—80 West Fortieth Street.